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Award Number: W81XWH-09-1-0550

TITLE: Role of the ARF Tumor Suppressor in Prostate Cancer

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CONTRACTING ORGANIZATION: The Washington University
                                                 Saint Louis, MO 63130

REPORT DATE: August 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-08-2011	2. REPORT TYPE Final	3. DATES COVERED *(From - To)* 1 AUG 2009 - 31 JUL 2011

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Role of the ARF Tumor Suppressor in Prostate Cancer	5b. GRANT NUMBER W81XWH-09-1-0550
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Leonard B. Maggi	5e. TASK NUMBER
E-Mail: lmaggi@dom.wustl.edu	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
The Washington University Saint Louis, MO 63130	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The nucleolar tumor suppressor ARF plays an important role in the tumor surveillance of human cancer. We have found that ARF expression is absent from highly proliferative prostate adenocarcinomas. This correlates with the normal expression of the p53 tumor suppressor gene indicating that ARF loss could be a contributing factor for prostate cancer initiation and/or progression. We have found that ARF-mull mice develop prostatic lesions by 9 months of age (2/10), but die of sarcoma or lymphoma. We have generated and are monitoring prostate specific ARF and ARF/p53 knockout animals for the development of prostate lesions avoiding the complication of genomic loss of these tumor suppressors. We have been unable to obtain a pure mouse prostate epithelial cell culture, therefore, we have taken two alternative approaches. First, we will knockdown basal ARF levels in commercially available normal human prostate epithelial cell lines. Second, we have developed a protocol to isolate polysomes from freshly isolated whole mouse prostates. Both of these techniques allow us to monitor polysome mRNA association in the absence of ARF. While we have encountered difficulties over the course of this two-year award, we have developed these new techniques to allow the research project to progress toward the approved goal of biomarker development and have applied for additional funding to continue the project.

15. SUBJECT TERMS

ARF, Prostate Cancer, Ribosome, Translation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	29	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION:

In numerous human cancers, the frequency of loss of the ARF tumor suppressor is second only to mutation of p53[1,2], providing critical evidence of ARF's role in preventing tumorigenesis throughout the body, irrespective of cell type. However, the result of ARF loss in the prostate is currently understudied. Our preliminary data using human samples from Washington University indicates that prostate adenocarcinomas typically maintain wild type p53 levels (97%), but lose ARF (96%) expression. These data suggest ARF loss may play a role in the development and/or progression of prostate cancer. Mechanistically, we have previously shown ARF to be a critical regulator of ribosome biogenesis[3-5].

The ultimate goal of this proposal is to identify new downstream therapeutic targets required for the development of prostate cancer through the identification of polysome-associated mRNAs in *Arf*[/-] prostate epithelial cells. *We hypothesize that the loss of Arf in prostate epithelial cells will lead to an increase in ribosome production and an alteration in the pool of polysome-associated mRNAs towards transcripts that encode proteins critical to prostate tumorigenesis. As such, these translated mRNAs will produce proteins that are required for prostate cancer development and therefore, these proteins will be potential novel targets for prostate cancer treatment or diagnosis.*

BODY:

As outlined in the approved Statement of Work, we focused our energies on the tasks planned for Months 1-24. These included experiments outlined in Tasks 1, 2, and 3. In this final progress report, we detail the progress and results from these studies.

Task 1. To determine the effects of *Arf* loss on Prostate Intraepithelial Neoplasia formation (Months 1-24):

a.   Examining the effects of prostate-specific *Arf* loss on prostate cancer formation and progression.

During the first year of the grant we sought to determine the effects of *Arf* loss on PIN formation in genomic *Arf*[/-] mice. Our preliminary studies performed in conjunction with the Washington University School of Medicine Small Animal Pathology Core were proven wrong due to tangential cuts in the prostate tissue sections examined. At the time of the grant submission we had promising preliminary results of 3 of 3 *Arf*[/-] and 0 of 4 wild type mice developing Prostate Intraepithelial Neoplasia (PIN). However, further submission of mice to the Small Animal Pathology Core found PIN lesions 6 of 7 *Arf*[/-] and 5 of 5 wild type mice. PIN lesions in the C57BL6 srtain of mice (which are the genetic background of our *Arf*[/-] mouse colony) have never been reported in the literature. We asked Dr. Jeff Arbeit in the department of Surgery here at Washington University for his expertise in mouse prostate tumor development. With his help, we have regenerated a cohort of 10 *Arf*[/-] and 12 wild type mice with the proper tissue section preparation as described[6]. Using the prostate "tree" layout, we were able to determine that 0 *Arf*[/-] and 0 wild type developed PIN (**Figure 1**). However, 2 of the *Arf*[/-] mice

developed prostatic lesions at 9 months of age (**Figure 2**). One grew in the ventral lobe of the mouse and was characterized by high levels of a proliferative marker, Ki-67. While the cells lacked basal (K5), luminal (K8), and neuroendocrine (synap) markers, the cells were Androgen Receptor (AR) positive (**Figure 2**, left column). The second lesion occurred in the anterior lobe of a different mouse. This lesion is appears to be a fibrosarcoma by hematoxylin and Eosin (H&E) staining of unknown origin as it does not stain for any of the prostate markers (**Figure 2**, right column).

As discussed in the original proposal, we anticipated a problem using male mice with a genomic loss of *Arf* who die by 9 months of age due to sarcoma or lymphoma development. We have produced a mouse colony containing males with a prostate specific deletion of *Arf* by crossing $Arf^{f/f}$ mice with ARR2Pb-Cre mice.   These Pb-Cre/$Arf^{f/f}$ and $Arf^{f/f}$ mice will be sacrificed between 15 and 18 months of age to assess prostatic lesion development and avoid the complications of sarcoma and lymphoma development. We have examined four (4) $Arf^{f/f}$, two (2) $Arf^{f/+}$ and two (2) Pb-Cre/$Arf^{f/f}$ mouse prostates by immunohistochemical staining at 18 months of age with no prostates demonstrating PIN or other lesions (**Figure 3**). This is not a problematic finding at this point as there may be a low penetrance of disease with only *Arf* loss. We will continue to examine the mouse prostates as more mice reach the proper age until we are statistically sure there is no phenotype. We currently have twenty-five (25) $Arf^{f/f}$ and four (4) Pb-Cre/$Arf^{f/f}$ male mice in the colony and are breeding to obtain more Pb-Cre/$Arf^{f/f}$ mice for the study.

In addition, we have produced a colony of Pb-Cre/$Arf^{f/f}$/$p53^{f/f}$ and $Arf^{f/f}$/$p53^{f/f}$ mice to examine for PIN and prostate cancer development.   One (1) Pb-Cre/$Arf^{f/f}$/$p53^{f/f}$ mouse was sacrificed at five (5) months of age and its prostate examined for PIN and other lesions. None were found (**Figure 4**) so we will continue aging the mice to at least 15 months before assessing their prostates for PIN. We currently have one (1) Pb-Cre/$Arf^{f/f}$/$p53^{f/f}$ and nine (9) $Arf^{f/f}$/$p53^{f/f}$ male mice, which we will allow to age to 15 months prior to sacrifice for PIN assessment. We will continue to produce litters to expand the number of male mice to assess for prostate cancer development as described above.

While we have made significant progress on this Task, we were unable to complete the assessment of our mouse model in only 2 years time. We had anticipated this as was mentioned in the original proposal. But with these new mouse models in hand, we are using the preliminary data to apply for more long term funding from the American Cancer Society through a Research Scholar Award and the National Institutes of Health through an R01 funded grant.

Task 2. To test the effects *Arf* loss on prostate epithelial cell growth (Months 1-9):

During the first year of the grant, we encountered problems with the murine prostate epithelial cell line we developed. While epithelial cells are present in the initial explants from freshly isolated mouse prostates (**Figure 5**, arrows) as demonstrated by positive staining for cytokeratin 8, e-cadherin (**Figure 6**), the population is overtaken by a stromal smooth muscle actin (SMA) positive cell population. The initial subpopulation of epithelial cells is luminal in origin as indicated by the Pan cytokeratin, cytokeratin 8, and E-cadherin positive staining and lack of basal and neuroendocrine markers cytokeratin 5 and synaptophysin; respectively (**Figure 6**).   As stated in the original proposal, these cells depend on androgens for growth as bicalutamide and flutamide, both inhibitors of Androgen Receptor (AR) signaling; inhibit their growth (data not shown). We have tried numerous culturing techniques including plating the

cells on collagen and PRIMARIA (Fisher Scientific) specialty tissue culture plastic. We have used Stem Cell Technologies' Mouse Prostate Epithelial Cell Isolation Kit to no avail. We have also combined techniques in various combinations with no success. For example, using the Mouse Prostate Epithelial Cell Isolation kit and plating the cells in Matrigel does not allow the epithelial cells to grow out from the stromal cells we continually see dominate the cultures. In addition, we have used several media formulations for epithelial and prostate cell culture. These have not helped to promote the specific growth of prostate epithelial cells in our cultures.

In our annual report from July 2010, we proposed one last technique to try to attain a pure prostate epithelial cell culture using a cell surface marker to sort the cell population to isolate only prostate epithelial cells. We used an Alexafluor-488 labeled E-cadherin antibody to isolate freshly isolated mouse prostate cells by FACS (Fluorescent Activated Cells Sorting). We tried several labeling procedures as recommended by different manufacturers with no success. We were able to isolate a small population of E-cadherin positive cells (data not shown), but they would not grow in culture. In addition, we tried to use a pan cytokeratin antibody, which allowed us to purify a population of cells as well, but these cells also failed to grow in culture (data not shown). Due to our lack of success after exhausting multiple approaches to obtain a pure murine prostate epithelial cell population, we decided to move ahead using the commercially available human prostate epithelial cells and p14ARF knockdown constructs as described below.

We have turned to normal human prostate epithelial cells, which are available from several commercial sources including Lonza (Basal, Switzerland) and Lifeline Technologies (Walkersville, MD). As shown in **Figure 7A**, p14ARF is expressed as very low levels in human prostate epithelial cells. This is not unprecedented as we have previously shown that wild-type mouse embryo fibroblasts (MEFs) have very low basal ARF that does have a role in regulating nucleolar function in the cell[5]. Therefore, the low level of ARF in normal human prostate epithelial cells (hPrEP) may be acting in a similar fashion. Using lentiviral siRNA constructs targeting p14ARF, we have tested the lentiviral siRNA constructs in a p14ARF positive cell line. As shown in **Figure 7B**, lentiviral constructs 9, 11, and 12 reduced the steady state mRNA levels of p14ARF. Due to the unique genomic structure of the CDKN2A locus, which harbors p14ARF (**Figure 8A**), we also examined the effects of knockdown on p16INK4A, which shares common exons 2 and 3 with p14ARF. As shown by western blot analysis in **Figure 8B**, only lentiviral constructs 6 and 7 are specifically directed against p14ARF (80% reduction each) and not p16INK4A. While these findings are different than the quantitative real time PCR results shown in Figure 7B, siRNAs do not always result in a decrease in mRNA levels. siRNAs have been shown to inhibit mRNA translation (reducing protein levels) without altering mRNA levels[7]. Therefore, the western blot data will guide which constructs we use.

a. What are the effects of *Arf* loss on ribosome biogenesis (Months 1-4)?

In addition to the lentiviral constructs, we also obtained p14ARF specific dsRNA oligos from IDT DNA (Coralville, IA). We chose to use LNCaP cells – a human prostate cancer cell line that contain similar levels of p14ARF as primary human prostate epithelial cells (**Figure 7A** compare lanes 1 and 2). These cells are easily transduced with siRNAs by nucleofection whereas the primary human prostate epithelial cells require viral infection to introduce exogenous DNA.

As shown in **Figure 9A**, p14ARF protein levels were reduced by 90% and 70% with si#1 and si#2, respectively, 48 hours post nucleofection. Using this transient form of p14ARF knockdown, we began working on the sub-Tasks of *Task 2* on the Statement Of Work.

Loss of p14ARF in LNCaP cells results in increased 47S pre-rRNA synthesis as measured by quantitative Real Time PCR (**Figure 9B**) indicating that p14ARF does indeed regulate basal rRNA transcription. We next examined steady state ribosome output by measuring cytoplasmic levels of rRNA separated over sucrose gradients. As shown in **Figure 9C**, loss of p14ARF also increases the steady state levels of polysomes (multiple ribosomes translating mRNAs) in LNCaP cells. Interestingly, there is no detectable change in the small (40S), large (60S) and monosome (80S) ribosome levels. Also, the degree of p14ARF knockdown (**Figure 9A**) correlates with the level of increase in actively translating ribosomes (**Figure 9C**). These data demonstrate that p14ARF regulates ribosome biogenesis in a human prostate cancer cell line, LNCaP.

b. What are the effects of *Arf* loss on protein synthesis and cell size (Months 5-6)?

The predicted result of increased ribosome biogenesis, as demonstrated in **Figure 9**, is increased cell mass and cell size. Knockdown of p14ARF in LNCaP cells results in increased protein content per cell as measured by Bradford Assay (**Figure 10A**). Interestingly, this does not result in an increase in cellular size (**Figure 10B**). Overall this is not inconsistent with the increase in cell mass measured in Figure 10A as cell size can be affected by many other factors besides protein production.

c. What is the dependence of the cellular growth on ARF expression (Months 6-9)?

This is another task that has caused problems. Due to the transient nature of p14ARF knockdown in LNCaP cells, it is not technically possible to do knockdown/rescue experiments to definitively prove p14ARF is controlling cellular growth in the LNCaP cell line. In addition, the primary human prostate epithelial cell lines are not easily manipulated once they are infected with lentiviral constructs (detailed below). The cells do not proliferate when passaged for further experiments (data not shown). Therefore, we have begun to create a knockdown/rescue construct to be able to knockdown p14ARF and express a p14ARF cDNA from the same lentiviral vector. We are using the pFRLu construct described by the Longmore laboratory[8]. We have successfully cloned the p14ARF si#1 sequence from IDT DNA (Figure 9A) into pFRLu. This siRNA sequence targets the 5'UTR (untranslated region) of p14ARF allowing the expression of the open reading frame of p14ARF, which lacks the 5'UTR. We are currently verifying the p14ARF open reading frame and siRNA cloning into pFRLu before continuing with these experiments.

Task 3. To determine the effect of loss of the *Arf* tumor suppressor on the pool of polysome associated mRNA in prostate epithelial cells. (Months 6-24)
    a. What are the effects of Arf loss on Polysome-Associated mRNAs in prostate epithelial cells (Months 6-9)?
    b. Validation of the mRNA's Identified in the Polysome Microarray (Months 10-16).
    c. Dependence of the Identified mRNA's on ARF expression (Months 14-18).
    d. Analysis of Validated mRNAs in prostate cancer tissues (18-24).

As we stated in our Annual Report, the work on this task was delayed due to the problems we had with the generation of the mouse prostate epithelial cell line. We planned on taking two approaches to perform the experiments detailed in the statement of work.

We described an approach to perform the polysome associated mRNA analysis on freshly isolated mouse prostates. Briefly, mice were cardiac perfused with PBS containing cycloheximide followed by RNA*later* (Applied Biosystems) containing cycloheximide to stabilize polysomes (ribosome-mRNA complexes) similar to what has been previously described[6]. One half (½) of the tissue corresponding to one of each of the 4 lobes (A, V, L, and D) was isolated, tamped to remove secretions, ground in a tissue grinder, and subjected to cytosolic polysome fractionation. The peaks corresponding to the polysome were collected and RNA isolated with RNA-solv (Omega-BioTek). Total cellular RNA was isolated from the other ½ of the prostate (1 lobe each A, V, L, and D).

As shown in **Figure 11A**, we were able to isolate polysomes from freshly isolated mouse prostates. Interestingly, while $Arf^{-/-}$ prostates had lower 40S, 60S, and 80S ribosome peaks compared to $Arf^{+/+}$, the polysomes in $Arf^{-/-}$ were significantly higher. Importantly, the difference between the translational profiles from *in vitro* cell culture and *in vivo* tissue is drastic (compare **Figures 11A** and **11B**). This data proves it is possible to measure polysome levels from freshly isolated mouse prostates.

However, we were concerned about the low levels of polysomes in both $Arf^{+/+}$ and $Arf^{-/-}$ samples. We decided to use this technique to examine the levels of polysomes in prostates isolated from mice, which have deletions of negative regulators of the mTOR (mammalian Target of Rapamycin) pathway (see **Figure 12**). PTEN is a negative regulator of PI3K (phosphoinositol-3 kinase), which is upstream of mTOR in the signaling pathway. PTEN-specific loss in the prostate leads to the development of metastatic prostate cancer[9]. We isolated prostates from 15-week-old $Pten^{+/+}$ and $Pten^{-/-}$ mice and subjected them to polysome analysis. As shown in **Figure 13A**, loss of *Pten* results in a dramatic increase in polysome levels. Interestingly, this profile from $Pten^{-/-}$ mice resembles the profile of cells in culture (compare **Figure 13A** to **Figure 11B**). This was encouraging as it demonstrated that we were able to obtain significantly more polysome-associated mRNAs compared to the $Pten^{+/+}$ mice from this technique.

We moved down the mTOR signaling pathway to examine a direct negative regulator of mTOR activation, *Tsc1* (**Figure 12**). Specific deletion of *Tsc1* in the prostate results in the formation of prostate adenocarcinoma, but only after 18 months[10]. Prostates isolated from these mice at 15 weeks of age did not result in a detectable change in polysome levels compared to $Tsc1^{+/+}$(**Figure 13B**). This was not completely unexpected, as at this age these mice have not developed prostate adenocarcinomas.

Taking into account the previous data from *Arf*, *Pten*, and *Tsc1* knockout mice, we next examined prostates from two other mouse models of prostate cancer. Deletion of the transcription factor Nkx3.1 leads to prostate intraepithelial neoplasia (PIN) after 25 weeks[11]. At 75 weeks of age, deletion of *Nkx3.1* leads to an increase in polysome formation *in vivo* (**Figure 13C**). In similar fashion, over prostate specific overexpression of Myc, a transcription factor known to regulated ribosome biogenesis[12], results in prostate tumorigenesis by 4 months of age[13]. Prostates isolated from Pb-Cre Myc (Hi-Myc) mice at 5 months of age have increased polysome profiles compared to wild-type littermate controls (**Figure 13D**). Similar to the $Pten^{-/-}$

prostates, *Nkx3.1*[−/−] and Hi-Myc prostates have a profile that closely resembles that of cells in culture.

Finally, we examined both *p53*^{−/−} and *Arf*^{−/−} prostate polysome profiles. Neither of these tumor suppressors has been examined in the context of prostate cancer initiation. Unfortunately, deletion of either gene alone does not lead to a dramatic increase in polysome formation. Repeating the data shown in **Figure 11A**, loss of *Arf* does lead to a significant increase in the polysome levels compared to wild-type controls (**Figure 13F**); however, loss of *p53* does not alter polysome levels from wild type levels (**Figure 13E**).

While disappointing these results are not completely negative. The three animal models which result in cell-culture-like polysome profiles (*Pten*, *Nkx3.1*, and *Tsc1*) also have actively growing tumors in their prostates. Normal prostates have very low proliferative levels as measured by BrdU (Bromodeoxy-Uridine) incorporation assays and Ki-67 immunofluorescent staining patterns[10]. These mouse models have very high levels of both proliferative markers. The three mouse models that do not show cell-culture-like profiles have wild-type levels of the BrdU and Ki-67 proliferative markers (data not shown). This correlation requires further investigation to understand what if anything it means for prostate tumor development.

Based on our initial findings in *Arf*^{−/−} prostates, we isolated RNA from whole prostates as well as from the polysome fractions. Quadruplicate samples for the polysome RNA and duplicate samples for total RNA were taken for Illumina Mouse 8 array analysis at the Microarray Core here at Washington University School of Medicine. We were unable to purify the polysome associated RNA well enough to perform the array. Using a Bioanalyzer to assess the quality of the RNA, we were unable to consistently obtain RNA of high enough quality to perform the microarray (data not shown). We also purified RNA from *Pten*^{−/−} whole prostates and polysome fractions as the *Pten*^{−/−} polysomes. We reasoned that the cell-culture-like profiles from *Pten*^{−/−} prostates would give us the best possible assessment of polysome-associated RNAs and the *Pten*^{+/+} would give us the lower threshold for this technique. The Bioanalyzer analysis for these samples (**Figure 14**) demonstrated this RNA was also of poor quality and therefore this method is not suitable for microarray experiments.

The other option we proposed in the Annual Report was to use the lentiviral siRNA mediated p14ARF knockdown in normal human prostate epithelial cells as described in *Task 2* above. Using normal human prostate epithelial cells lacking p14ARF, we wanted to perform the polysome associate RNA Microarray. However, we ran into the problems in obtaining enough cells to perform the experiments. Once infected with the lentiviral constructs, the cells would not survive expansion in cell culture. We have attempted several means of trypisinizing, washing, and pelleting the cells with little success (data not shown). We are attempting to work out the proper conditions to infect the cells and harvest directly for the polysome assay. Once we have worked out the proper conditions, we will be able to continue the work on this task with primary human prostate epithelial cells.

Given our success in knocking down p14ARF in LNCaP cells and being able to analyze them in multiple ways, we attempted to perform our polysome mRNA analysis using this model system. As shown in **Figure 9C,** knocking down p14ARF results in an increase in polysome levels. We repeated this experiment and used the isolated polysome-associated RNA to create a 150 bp cDNA library for deep sequencing. In collaboration with Dr. John Edwards here at Washington University School of Medicine, we have the ability to use this new technology to obtain a complete record of the mRNAs associated with the polysome upon p14ARF loss. This is a much better technique than the original Microarray approach proposed, as we are not limited

to the number and quality of the probes provided. This will give us a complete picture of all the mRNAs that move onto and off of the polysome upon p14ARF loss in LNCaP cells. This does not alter the approved Statement of Work as we are using a more robust technique to obtain the list of potential biomarkers. As shown in **Figure 15**, we have produced cDNA libraries from the polysome-associated mRNAs in LNCaP cells in the presence and absence of p14ARF. We have sent these libraries to the GTAC Deep Sequencing Core here at Washington University School of Medicine and are waiting for their analysis of the preparations before moving forward with the sequencing. Once we have our data, Dr. Edwards has a data analysis pipeline in place to help us identify the p14ARF-regulated mRNAs. We will then be able to validate the hits and move forward with our biomarker panel.

KEY RESEARCH ACCOMPLISHMENTS:

- We have determined that $Arf^{-/-}$ mice develop prostatic lesions (2 of 10 $Arf^{-/-}$ vs. 0 of 10 $Arf^{+/+}$) at 9 months of age, but die from lymphoma or sarcoma at that time.
- We have developed four new mouse colonies for prostate cancer modeling – Pb-Cre/$Arf^{f/f}$, Pb-Cre /$p53^{f/f}$, Pb-Cre/$Arf^{f/f}$/$p53^{f/f}$, and $Arf^{f/f}$/$p53^{f/f}$ which will allow the mice to be followed beyond the lifespan of the corresponding genomic knockouts.
- We have begun to accumulate data from our Pb-Cre $Arf^{f/f}$ and Pb-Cre $Arf^{f/f}$/$p53^{f/f}$ models of prostate cancer (0 of 2 Pb-Cre $Arf^{f/f}$ vs. 0 of 4 $Arf^{f/f}$ and 0 of 1 Pb-Cre $Arf^{f/f}$/$p53^{f/f}$).
- We have developed a wild-type and $Arf^{-/-}$ SMA-positive prostatic stromal cell line to use as a tool to assess stromal contributions to prostate cancer development.
- We have identified 3 dsRNA oligos as well as 2 lentiviral shRNA constructs to use to knockdown p14ARF in human prostate epithelial cells.
- We have developed a new protocol for examining polysome levels from freshly isolated mouse prostates.
- We have generated cDNA libraries from polysome-associated mRNAs in control and p14ARF-knockdown human prostate cell lines, which have been sent to the GATC (Washington University School of Medicine Core Facility) for Illumina-based Deep Sequencing.

REPORTABLE OUTCOMES:

Manuscripts/Abstracts:
Maggi, LB, Jr. Deregulation of Ribosome Biogenesis and mRNA Translation by Loss of the ARF Tumor Suppressor. *IMPaCT Conference 2011 Proceedings* pg 258 (2011)

Winkeler, CL, Kladney, RD, **Maggi, Jr.**, LB, and Weber, JD. 2011. Cre-mediated excision of floxed alleles in the gametes of the bone-specific CtsK-Cre mouse. *In review at J Bone and Min Res.*

Presentations:
"Protein Translation and Prostate Cancer"   Prostate Cancer Research Group
                                                   Washington University School of Medicine
                                                   Saint Louis, MO (11/05/2009)

"Arf Loss Alters the Translatome to Permit   Breast Cancer Research Group
Transformation   "   Washington University School of Medicine
                                                 Saint Louis, MO (11/13/2009)

"Polysome Associated mRNA – Priming Cells   Microarray Workgroup
for Transformation"   Washington University School of Medicine
                                                 Saint Louis, MO (12/04/2009)

"The mTOR Pathway: Translational Control   Cancer Biology Pathway
and Cancer."   Washington University School of Medicine
                                                 Saint Louis, MO (01/26/2010)

Patents/Licenses: None

Animal Models:
Pb-Cre/*Arf*$^{f/f}$– Specifically deletes the *Arf* tumor suppressor in the prostate allowing the mice to be followed for the development of prostate lesions beyond the 9-month lifespan of genomic *Arf*$^{/-}$ mice.

Pb-Cre/*p53*$^{f/f}$ – Specifically deletes the p53 tumor suppressor in the prostate allowing mice to be followed for the development of prostate lesions beyond the 5-month lifespan of genomic *p53*$^{-/-}$ mice. These mice have been reported in the literature, but this is a newly developed colony at Washington University.

Pb-Cre/*Arf*$^{f/f}$/*p53*$^{f/f}$– Specifically deletes the *Arf* and *p53* tumor suppressors in the prostate allowing the mice to be followed for prostate lesion development beyond the lifespan of genomic knockouts of either gene alone.

Arf$^{f/f}$/*p53*$^{f/f}$ – Wild type control mice for experiments using Pb-Cre/*Arf*$^{f/f}$/*p53*$^{f/f}$ mice.

Applied for Funding:
2010 Department of Defense Peer Reviewed Cancer Research Program Idea Award -
        "Neurofibromin-regulated Protein Translation in NF1" (CA100049)
        Role – PI (95% Effort)
        Result - *Rejected*

2010 Department of Defense Prostate Cancer Research Program Exploration: Hypothesis
        Development Award – "Translational Alterations in c-Myc Driven High Grade PIN
        Lesions In Vivo" (PC100153)
        Role – PI (95% Effort)
        Result - *Rejected*

2010   Siteman Cancer Center Washington University Developmental Research Award in
        Prostate Cancer – "Translational Alterations in in c-Myc Driven High Grade PIN
        Lesions In Vivo"
        Role – PI (95% Effort)

Result - *Rejected*

2011   NIH R01 – Cancer Molecular Pathobiology Study Section – "Mechanisms of Transformation in T(4; 14)+ Myeloma" (1 R01 CA164240-01)
        Role – Co-Invesitgator (10% effort)
        Result – *44th percentile* (Will resubmit in November 2011)

2011   Komen Career Catalyst Award – "ARF-regulated Translational Priming for Oncogenesis"
        Role – PI (25 % Effort)
        Result - *Pending*

2011   American Cancer Society – Research Scholar Grant – "ARF-regulated Translation Priming for Oncogenesis"
        Role – PI (25% Effort)
        Result - *Pending*

2011   Concern Foundation – CONquer CanCER Now Award – "The ARF-NPM Network Regulates Survival Through Ribosome Production in Cancer"
        Role – PI (10% Effort)
        Result - *Pending*

Promotion:
January 1, 2011   Research Assistant Professor at Washington University School of Medicine

Research Opportunities:
2010   Siteman Cancer Center, Research Associate Member
2010-   Institute of Clinical and Translational Sciences, Washington University, Member
2011-   AAAS, member
2011- 2014   Elected Clinical Representative to the Executive Committee of the Faculty Council, Washington University School of Medicine

CONCLUSIONS:

During the two-year span for this project, we have made significant progress on the outlined Tasks. While it has been difficult, we have found alternative approaches for each of the problems encountered and have made significant progress toward our goal of biomarker discovery. These alternative approaches did not alter the approved statement of work. They only altered the starting material for the experiments using human prostate epithelial cell lines instead of mouse prostate epithelial cell lines. While we are not happy about all the roadblocks that came up over the course of this award, we have been able to address them and move forward. This award has established a foundation for future work and new grants have already been prepared and submitted.

We have shown that 20% of $Arf^{-/-}$ mice develop prostatic lesions. However, these mice die at 9 months of age due to disease unrelated to the prostatic lesions. To circumvent this problem, we

have developed four (4) new mouse models for prostate tumor formation, Pb-Cre/$Arf^{f/f}$, Pb-Cre/$p53^{f/f}$, $Arf^{f/f}/p53^{f/f}$, and Pb-Cre/$Arf^{f/f}/p53^{f/f}$. These prostate specific animal models will allow us to assess ARF's specific role in prostate tumorigenesis. These studies will continue as we obtain the appropriate numbers of mice to assess the impact of *Arf* and *p53* on prostate tumorigenesis.

We have developed a new technique to assess the role of ARF in polysome formation *in vivo*. Using freshly isolated mouse prostates, we can assess *in vivo* levels of polysomes upon ARF loss. Importantly, our data shows that *in vivo* polysomes are drastically different that those reported for cell culture. The homeostatic translational profile is much lower in tissues relative to continually proliferating cell culture. Unfortunately, we have not been able to produce high quality polysome-associated RNA from these samples. This has left us to just assess the translational levels in mouse prostates.

We have found 3 siRNA oligos and 2 lentiviral constructs that can be used to knockdown *Arf* mRNA expression in human prostate epithelial cells. These have allowed us to assess for the first time ARF's role in ribosome biogenesis in human cells. Additionally, these constructs have allowed us to perform polysome associated mRNA deep sequencing to assess ARF's effect on the proteins produced in human prostate cells. We are close to obtaining a preliminary list of biomarkers from the polysome-associated mRNA deep sequencing, which we will begin to validate.

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**APPENDICIES:**   None

SUPPORTIN DATA: See below (Figures 1-15)
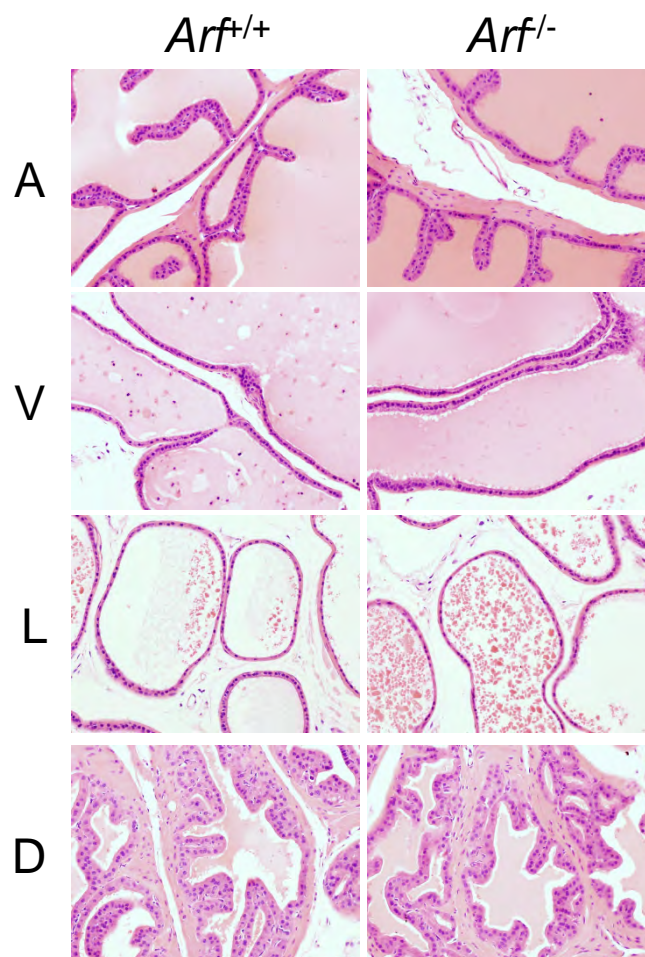
Figure 1.   Representative H&E Stain of *Arf*^{+/+} and *Arf*^{-/-} Mouse prostates.   Thirty-nine (39) week old mice were sacrificed and their prostates removed, fixed, sectioned, and stained with Hematoxylin and Eosin. 20x magnification; A, Anterior; V, Ventral; L, Lateral; D, Dorsal.
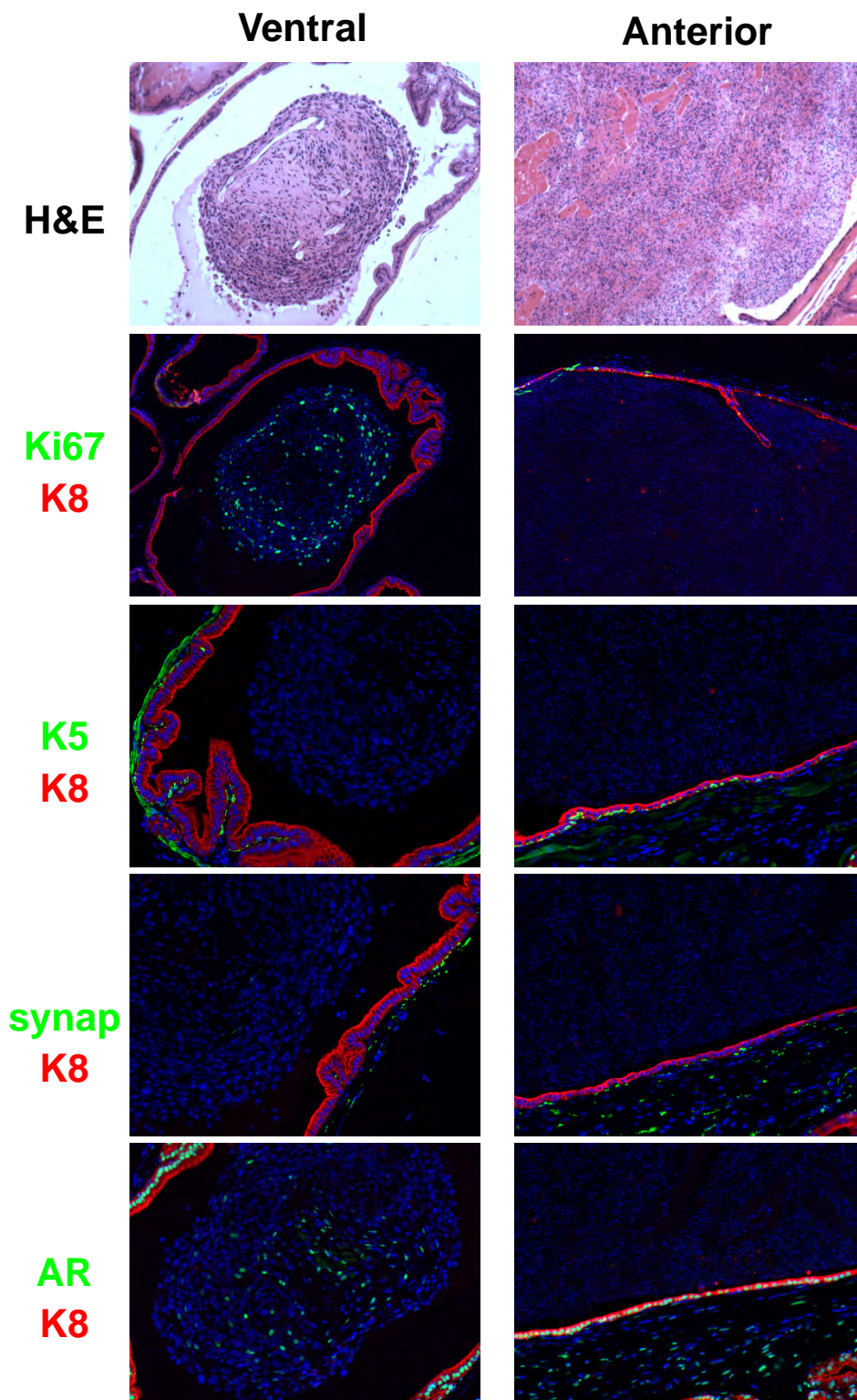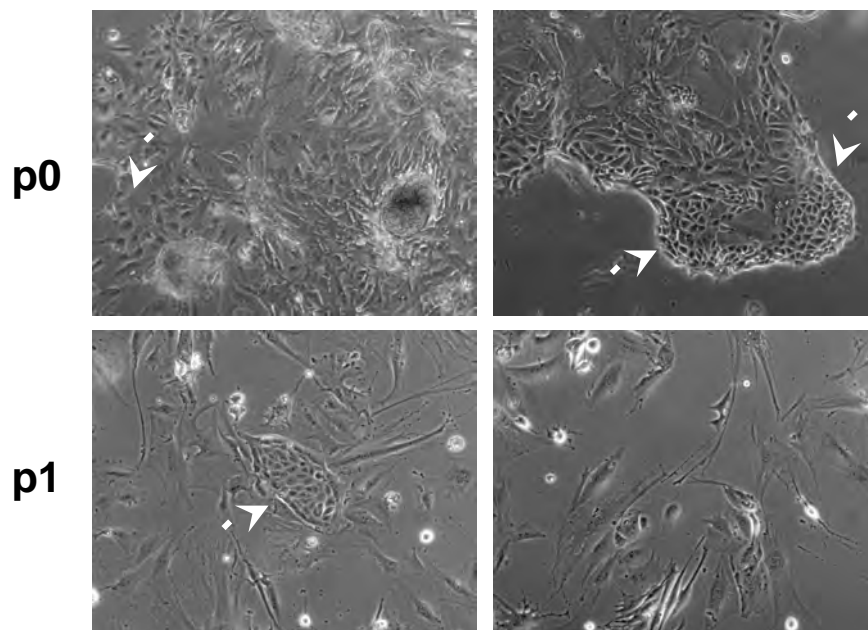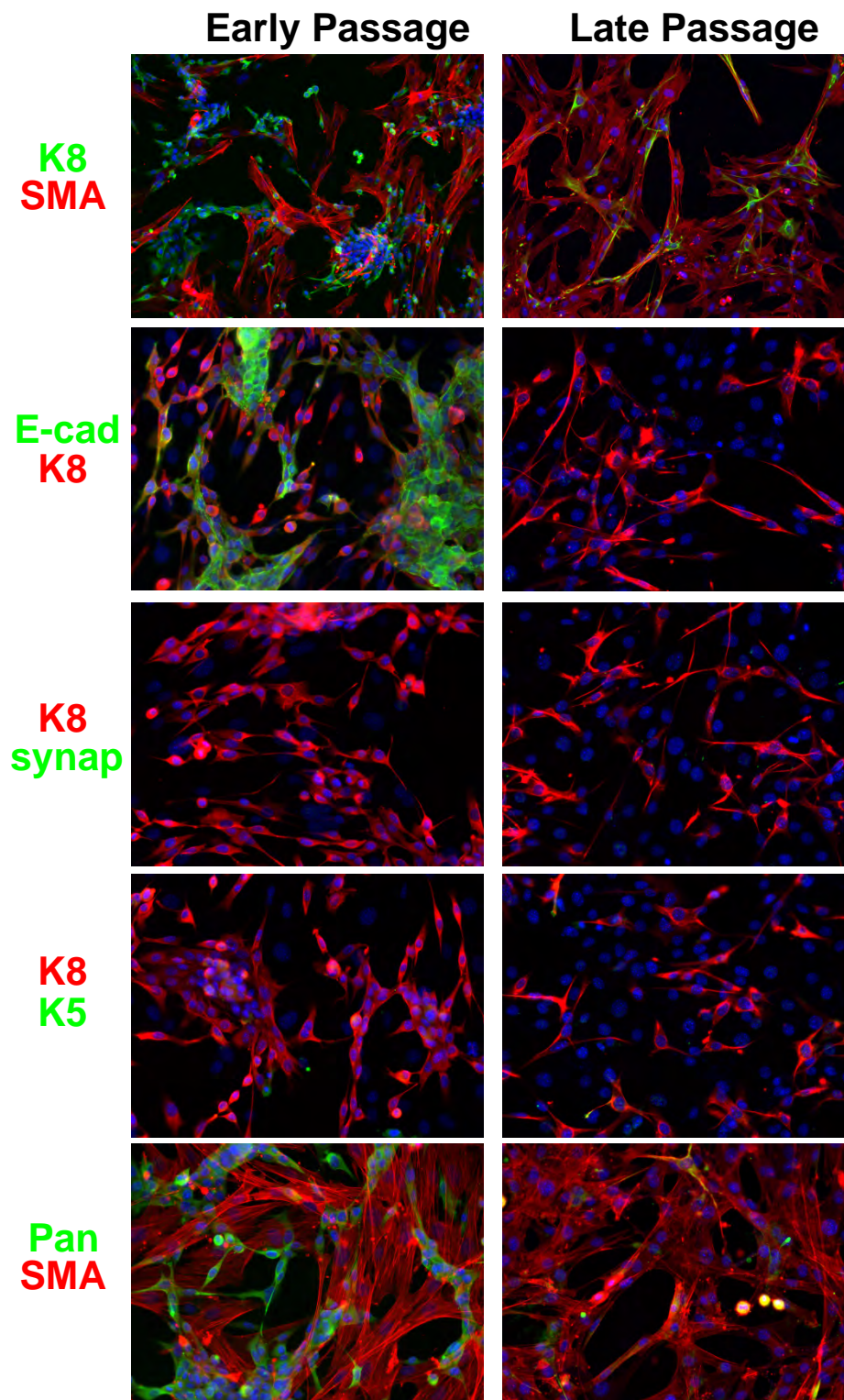
Figure 2. Characterization of *Arf⁻/⁻* Prostate Lesions. Thirty-nine (39) week old mice were sacrificed and their prostates removed, fixed, sectioned, and stained as indicated. One mouse had a ventral lesion and the second had an anterior lesion. Nuclei are stained with DAPI (blue) except Hematoxylin & Eosin (H&E) panels.   K8, cytokeratin 8; K5, cytokeratin 5; synap, Synaptophysin; AR, Androgen Receptor.
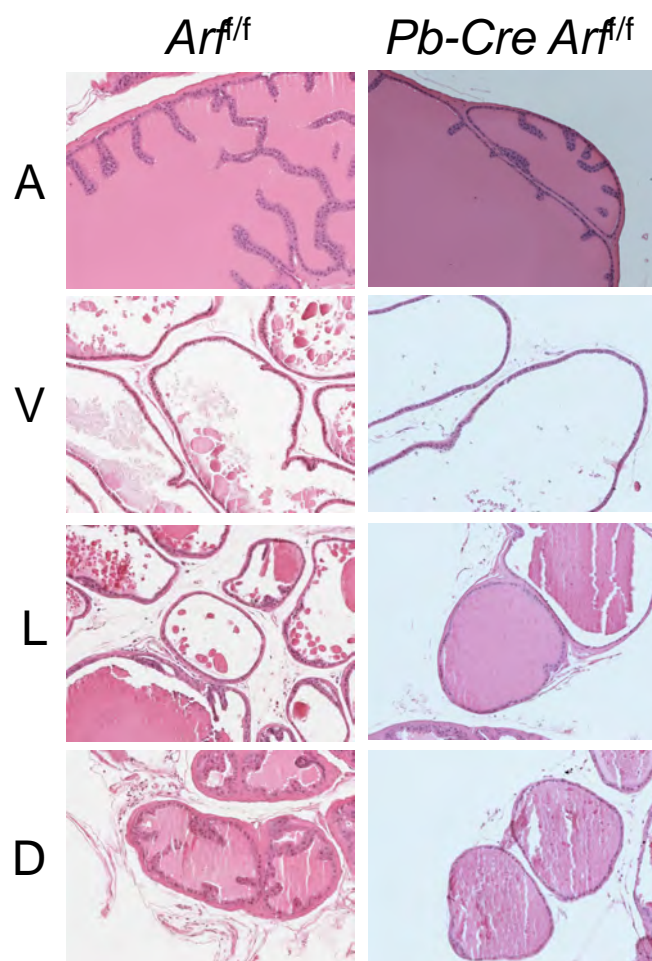
Figure 3. Representative H&E Stain of *Arf*f/f and Pb-Cre *Arf*f/f Mouse prostates. Eighty (80) week old mice were sacrificed and their prostates removed, fixed, sectioned, and stained with Hematoxylin and Eosin. 10x magnification; A, Anterior; V, Ventral; L, Lateral; D, Dorsal.
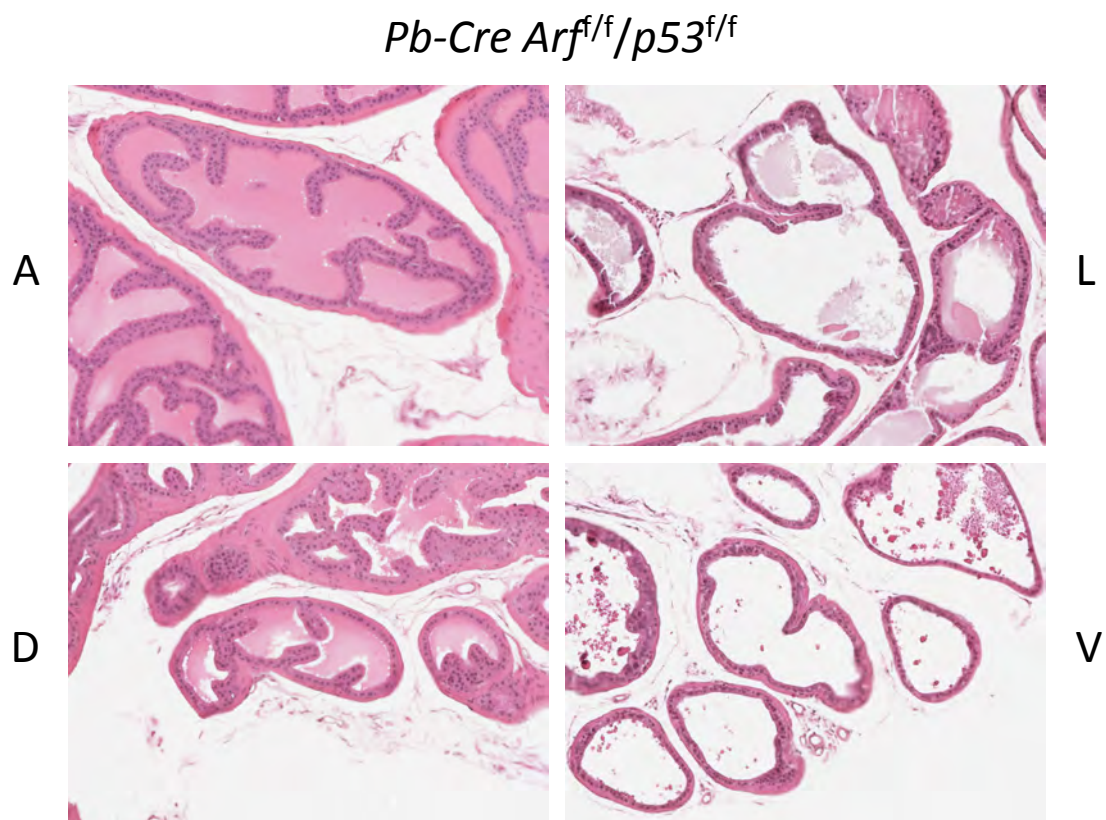
## *Pb-Cre Arf*^{f/f}/*p53*^{f/f}

Figure 4. H&E Stain of a Pb-Cre *Arf*^{f/f}/*p53*^{f/f} Mouse prostate.   A twenty-one (21) week old mouse waw sacrificed and its prostate removed, fixed, sectioned, and stained with Hematoxylin and Eosin. 10x magnification; A, Anterior; V, Ventral; L, Lateral; D, Dorsal.

Figure 5.   Characterization of *Arf*^{-/-} Prostate Cell Cultures.   Phase contrast microscopy of freshly explanted cultures (p0) show a mixed population of cells with epithelial cells present (arrow). A single passage (p1) results in a loss of epithelial cell morphology in the cultures.

Figure 6.   Characterization of *Arf^-/-* Prostate Cell Cultures.   Early passage (<6) and late passage (>12) *Arf^-/-* mouse prostate cells were fixed in formalin and stained for the indicated prostate epithelial markers (K8, cytokeratin 8; SMA, smooth muscle actin; E-cad, E-Caherin; K5, cytokeratin 5; synap, Synaptophysin; Pan, Pan cytokerain. All panels have nuclei stained with DAPI. 10X magnification.

A

B
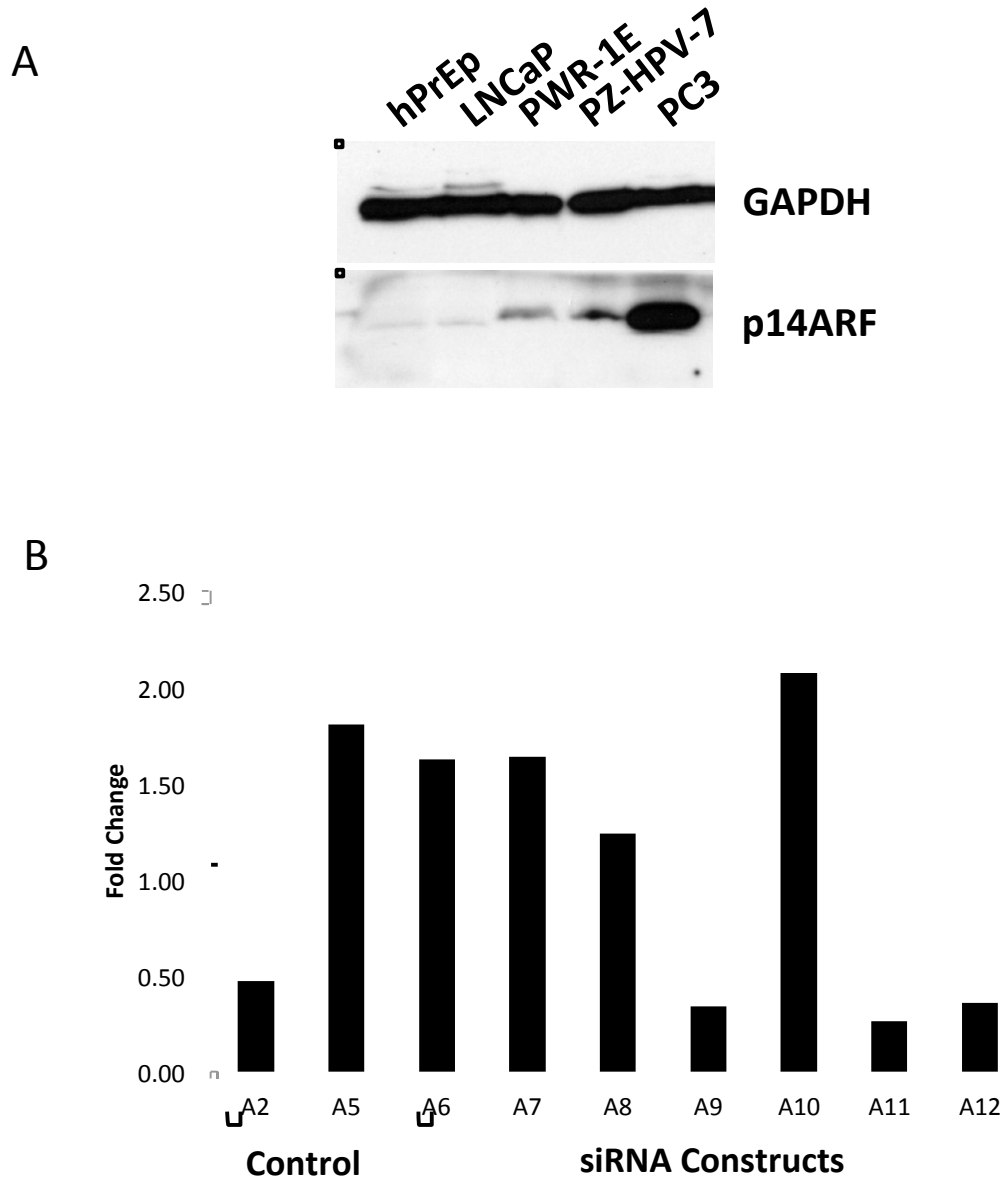
Figure 7. p14ARF levels in human prostate cell lines. (A) Western blot analysis of p14ARF protein levels in human prostate epithelial cell lines. hPrEP, normal human prostate epithelial cells. **(B)** Real Time PCR analysis of p14ARF mRNA levels normalized to HPRT and relative to uninfected cells upon lentiviral delivered siRNA knockdown. Fold Change is calculated by the $2^{-\Delta\Delta Ct}$ method.

A

B
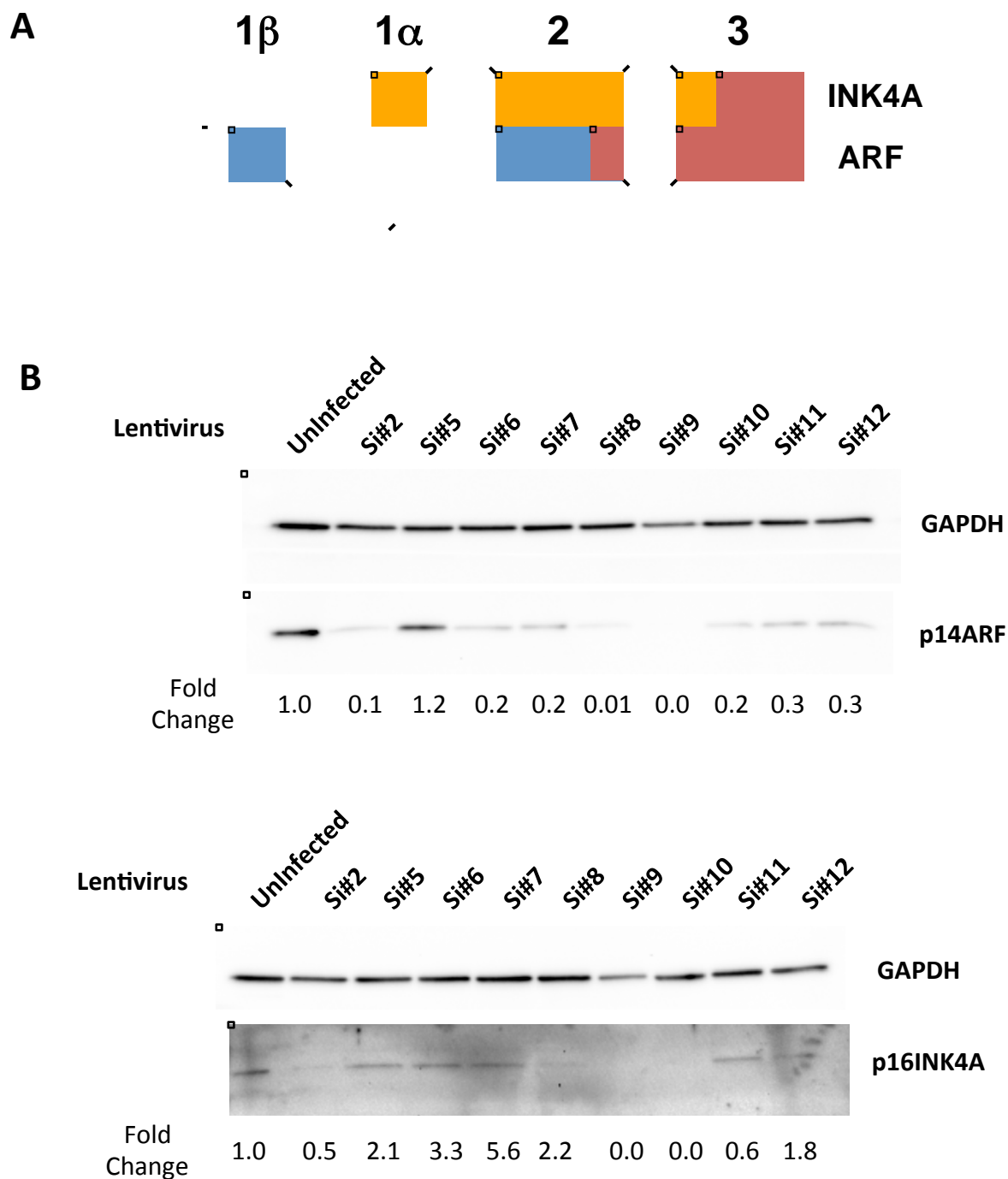
Figure 8. Specificity of p14ARF knockdown. A Schematic of the CDKN2A locus showing separate first exons 1a for p16INK4A and 1b for p14ARF splicing into common exons 2 and 3. **B** Cells infected with the indicated lentiviral constructs were harvested 72 h post infection and assessed for p14ARF and p16INK4A expression.

A

	siCTL	si#1	Si#2
GAPDH			
p14ARF			

Fold Change   1.0   0.1   0.3

B

C

[Sucrose]
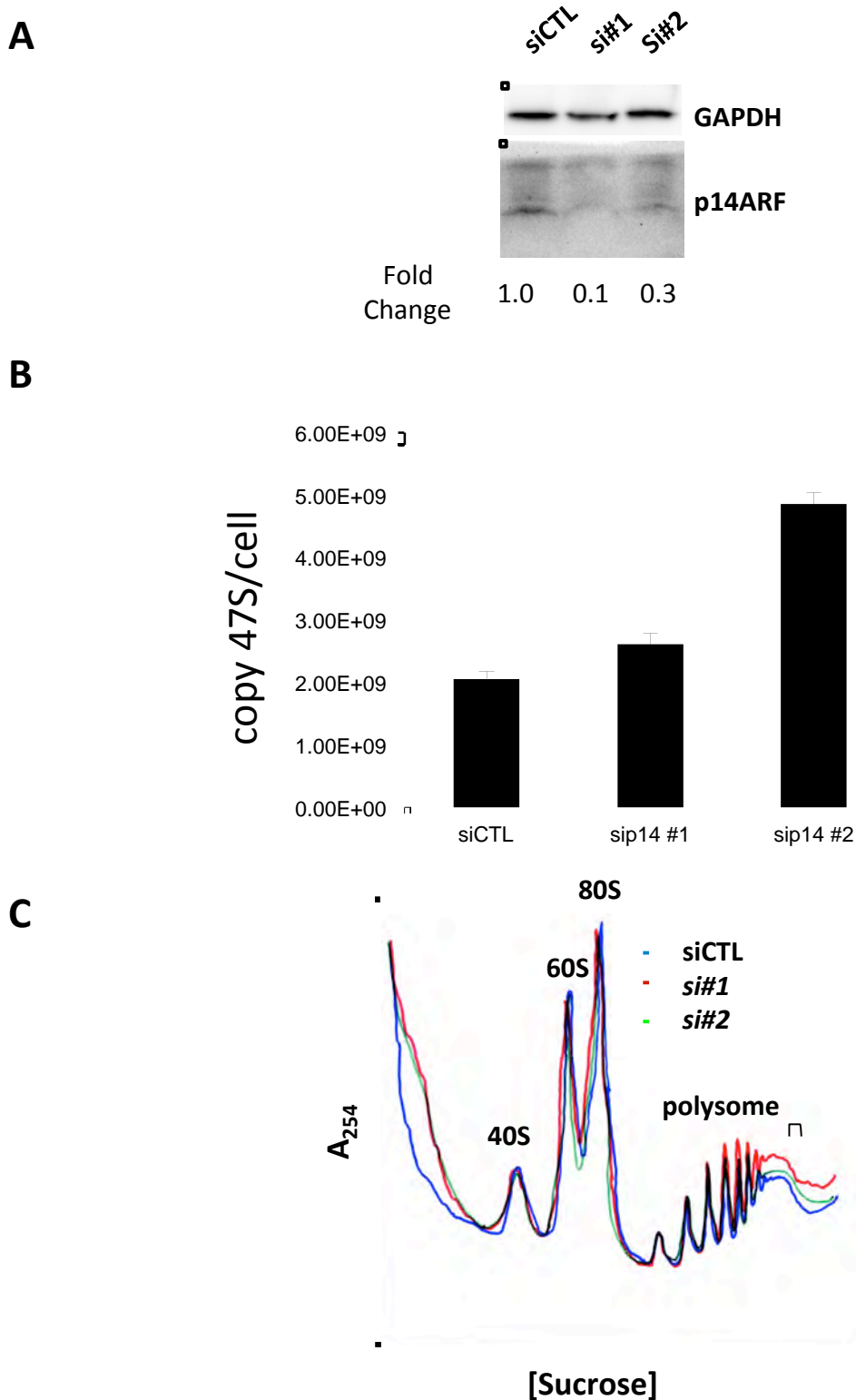
Figure 9. p14ARF knockdown in LNCaP cells results in increased rRNA transcription and protein synthesis. LNCaP cells were nucleofected with the indicated siRNA constructs and harvested 48 h later. **A** Western blot analysis of p14ARF levels. **B** Total RNA was isolated and analyzed by SYBR Green quantitative PCR for 47S pre-rRNA transcript copy number. Results are means ± SD. **C** Cell were harvested, counted and cytoplasmic extracts separated on sucrose gradients. The gradients were fractionated with constant monitoring at 254 nm to measure RNA.

A

B
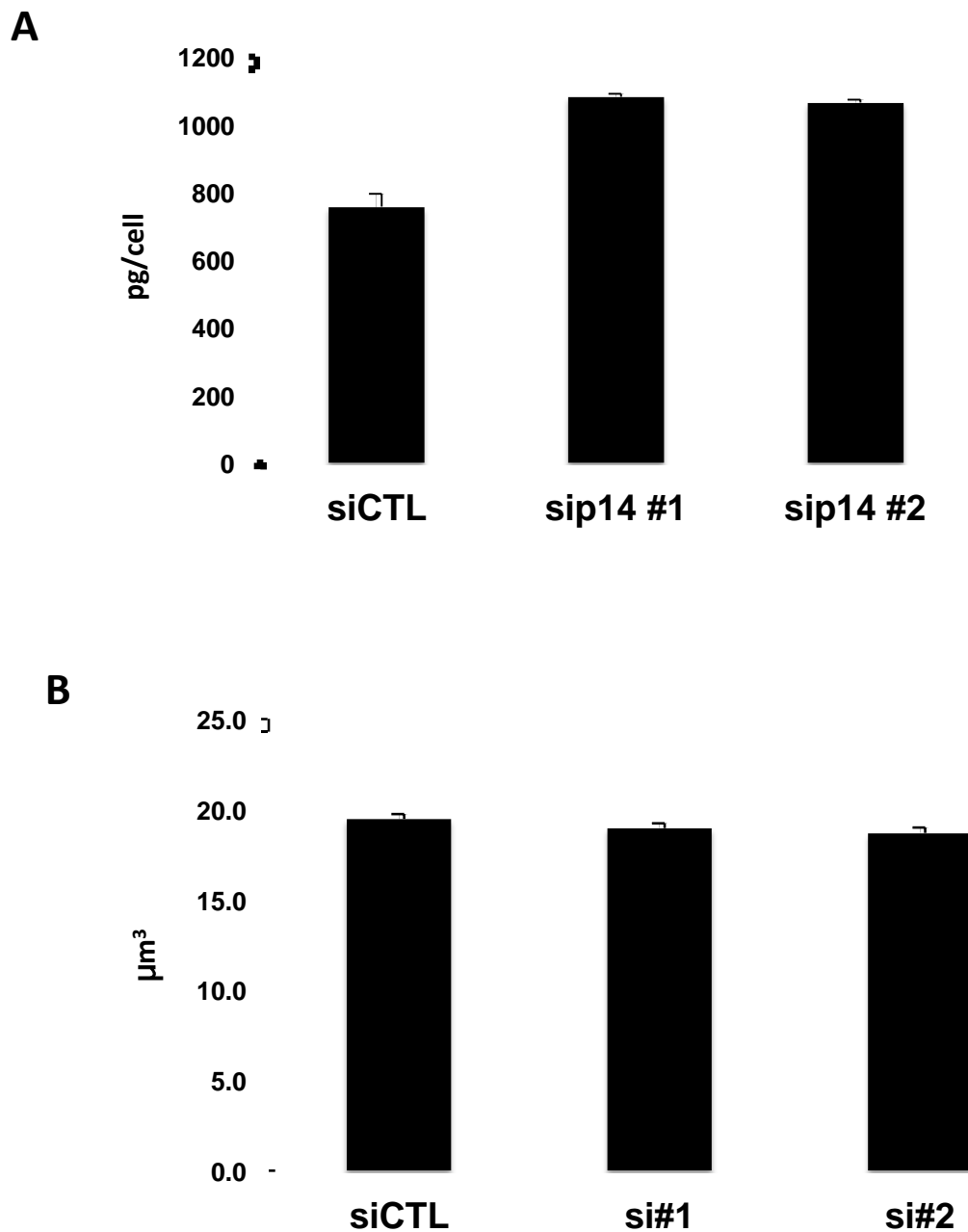
Figure 10. Loss of p14ARF increased cell mass and size. A Cell were harvested, counted and lysed, and total protein measured by Bradford assay. Results are means ± SD. **B** Cells were harvested and analyzed for cell diameter with a Nexelcom Cellometer. Results are means ± SEM.
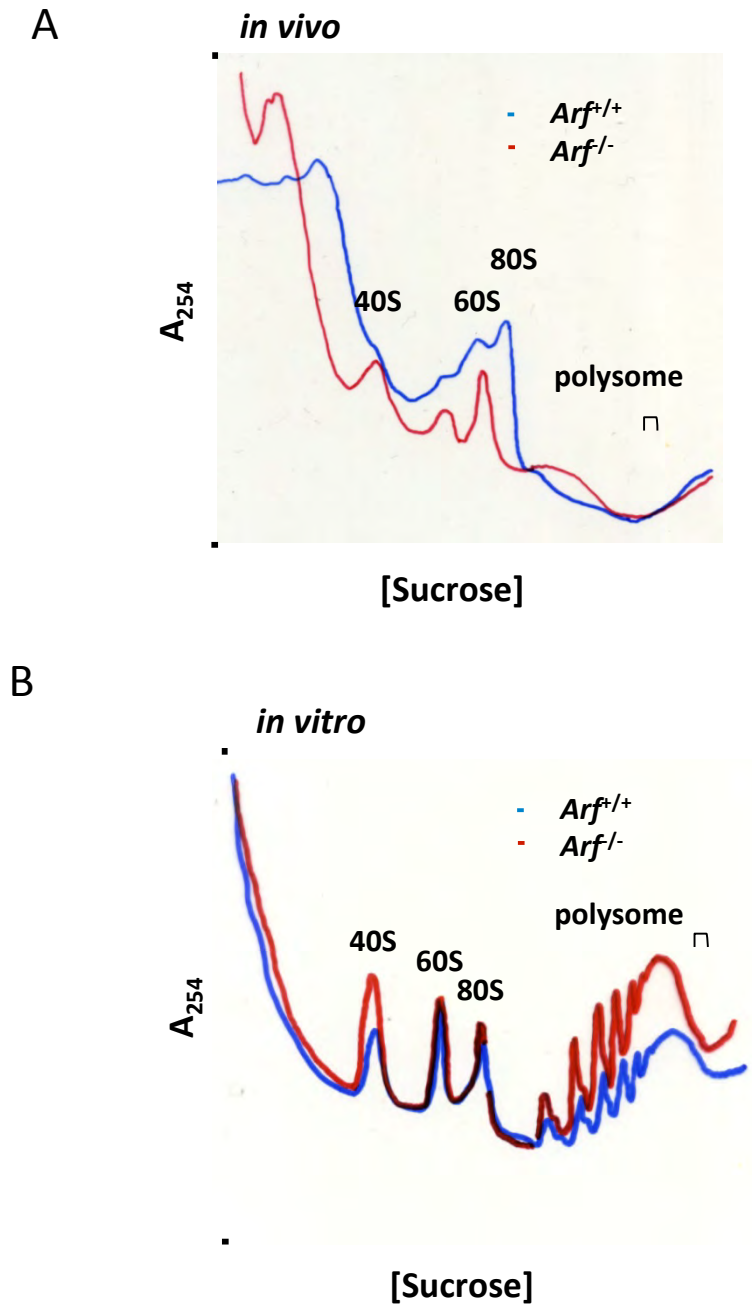
Figure 11. Effects of ARF loss on polysome formation *in vivo*. (A) Cytosolic ribosome formation was measured on freshly isolated wild type and *Arf*-/- mouse prostates. One-half of each prostate consisting of 1 lobe each, Anterior, ventral, lateral and dorsal was subjected to polysome analysis. **(B)** Cytosolic ribosome formation was measured on equal numbers (3x10^6) of wild type and *Arf*-/- mouse embryo fibroblasts.
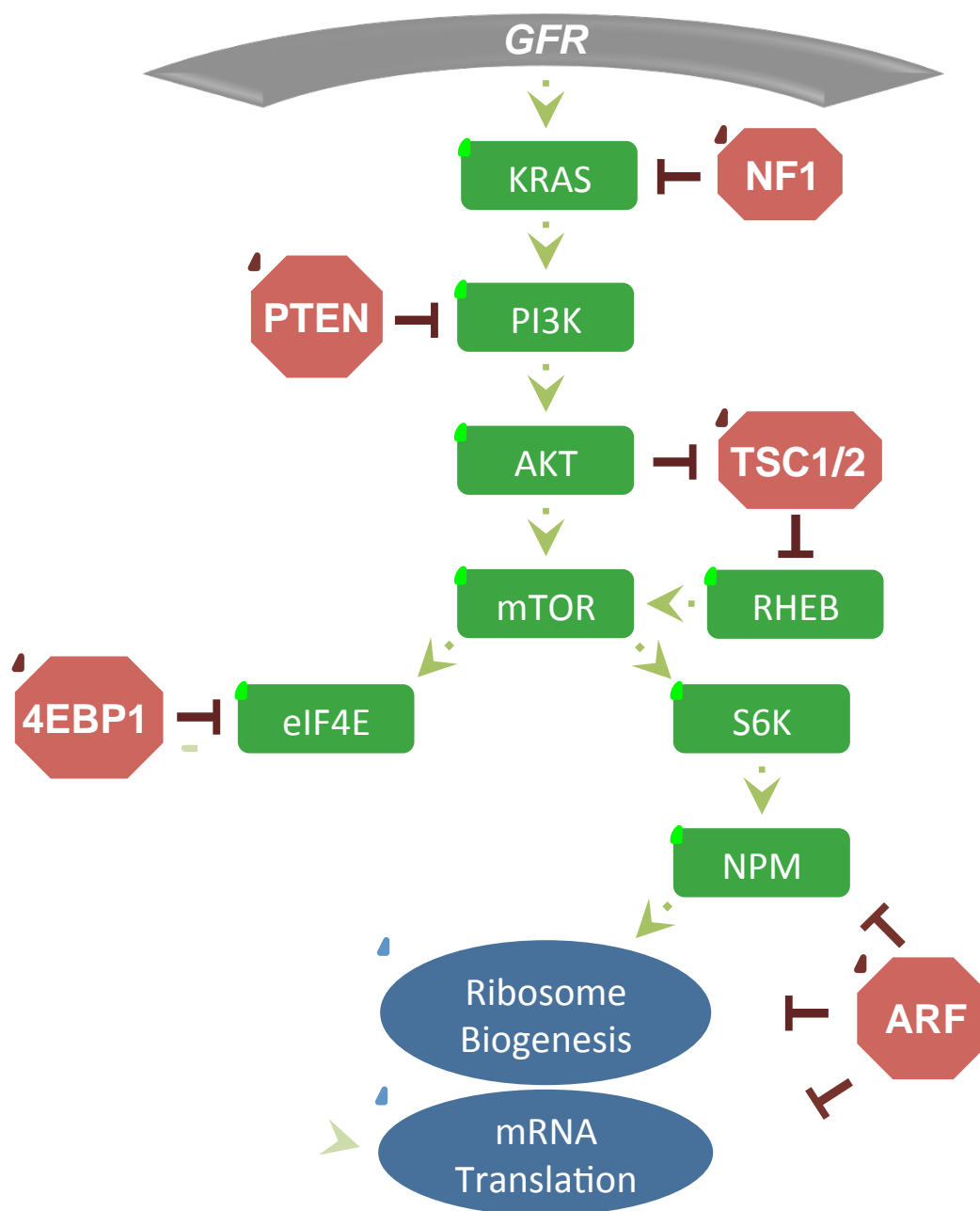
Figure 12. The mTOR Signaling Pathway.   Activators of Ribosome Biogenesis and mRNA translation are shown in green and inhibitors are shown in red.
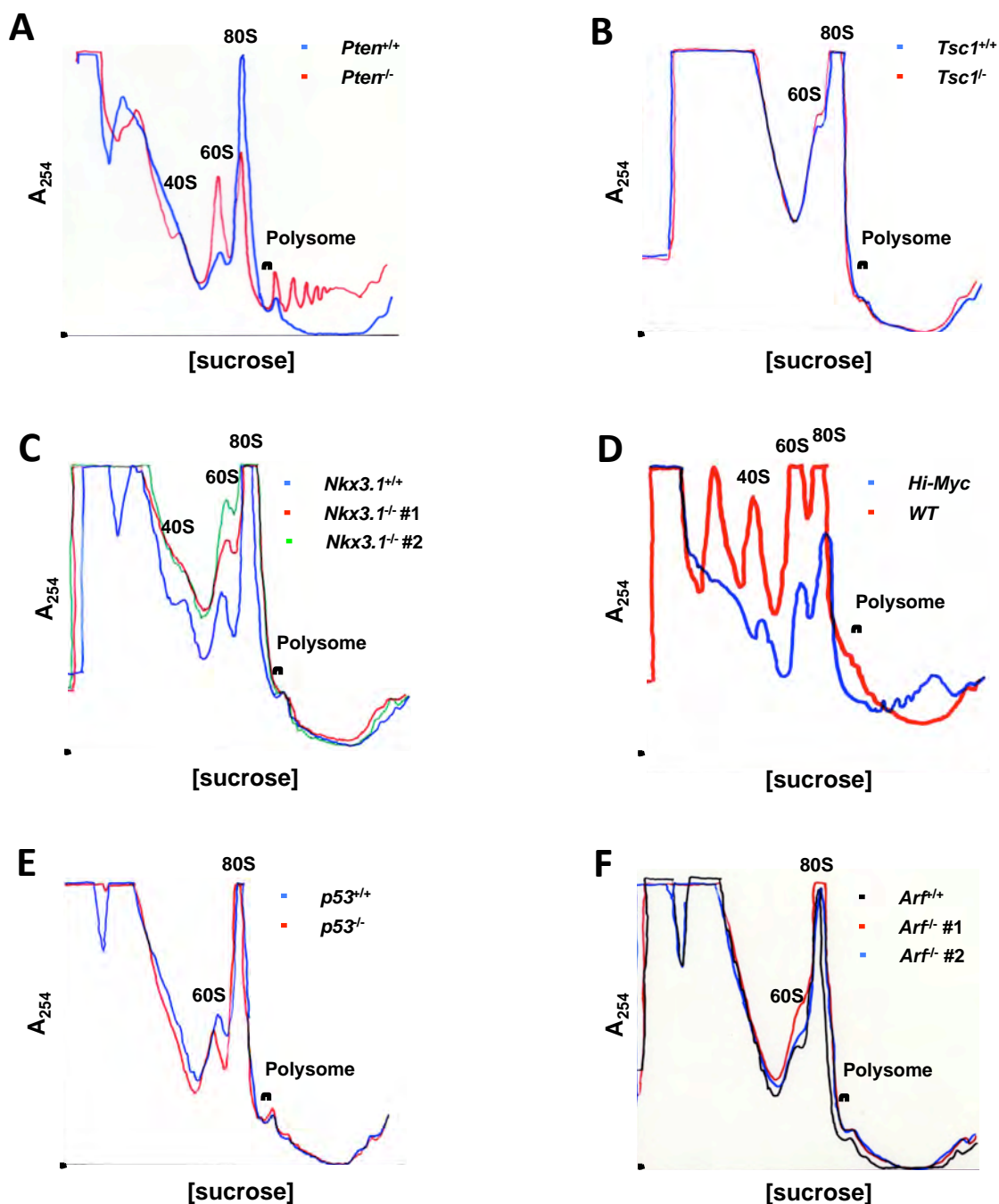
Figure 13. Mouse Prostate *in vivo* Polysome Profiles. Prostates from the indicated genotypes of wild-type and knockout mice were isolated, cytoplasm extracted, and separated over sucrose gradients. The gradients were fractionated with constant monitoring at 254 nm to measure rRNA content. The traces are representative of at least 3 isolations per genotype.
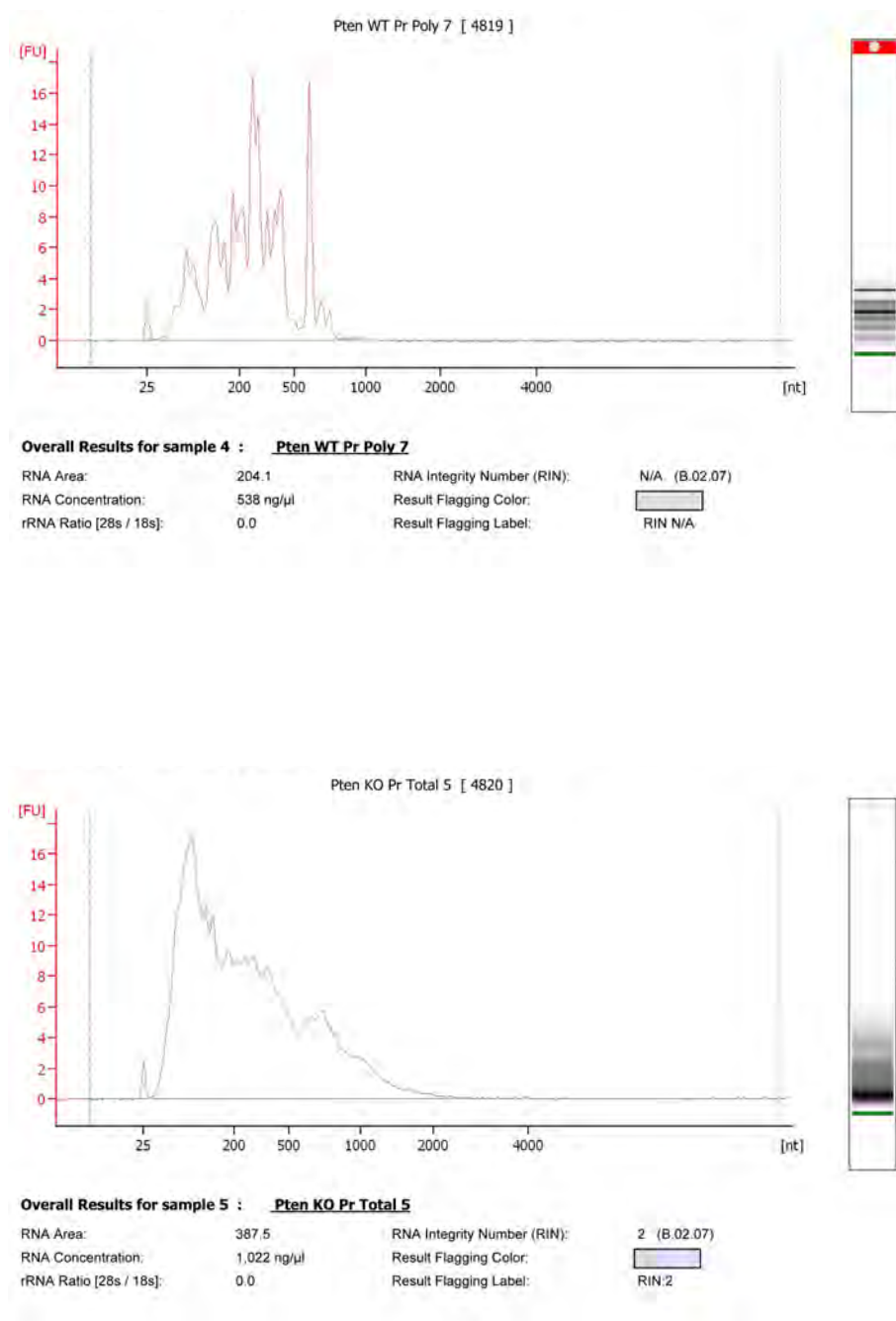
Figure 14. Representative Bioanalyser data from *Pten*^{+/+} and *Pten*^{-/-} polysome RNA samples.
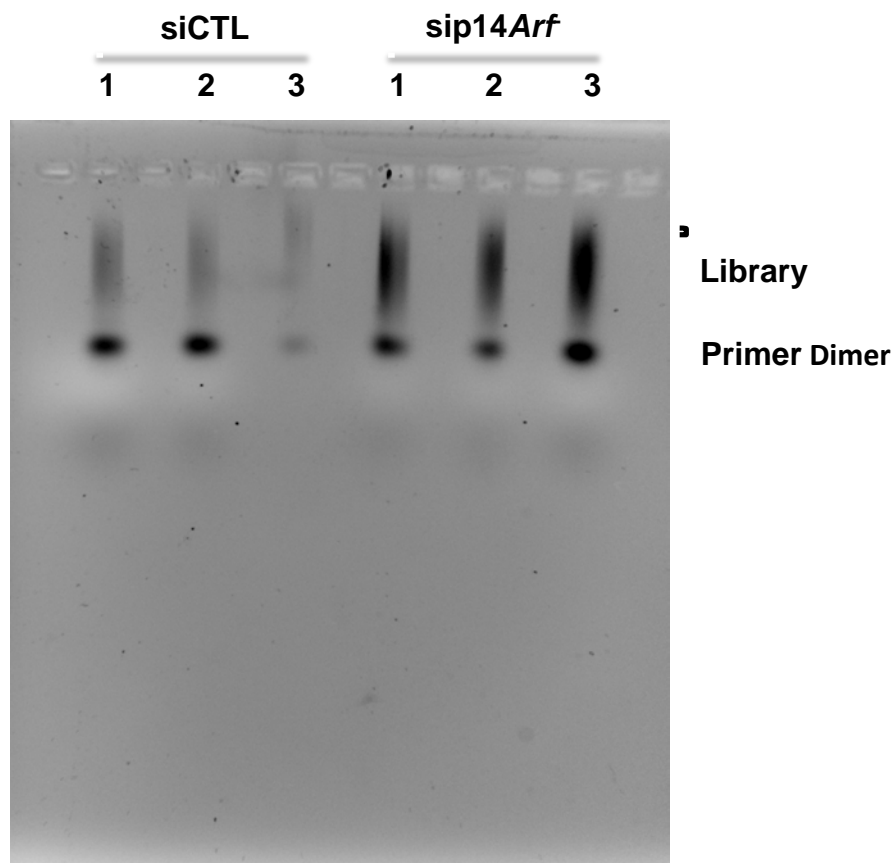
Figure 15. Example of Deep Sequencing Library Prep. Triplicate samples of polysome-associated mRNA libraries from LNCaP cells are shown.